# PARTIAL CHARACTERIZATION OF CYCLODEXTRIN GLUCANOTRANSFERASE BY *Bacillus* sp. TS1-1

UMMI NOR HUSNA HUSIN

A thesis submitted in fulfillment
of the requirements for the award of the degree of
Bachelor of Chemical Engineering (Biotechnology)

Faculty of Chemical & Natural Resources Engineering
Universiti Malaysia Pahang

MAY 2009

# DECLARATION

I declare that this thesis entitled "Partial Characterization of Cyclodextrin Glucanotransferase by *Bacillus* sp. TS1-1" is the result of my own research except as cited in references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree."

| | | |
|---|---|---|
| Signature | : | ……. |
| Name | : | Ummi Nor Husna Husin |
| Date | : | May 2009 |

# DEDICATION

*Special dedication to*
*Rahana Mahmud my beloved mother and*
*Husin Ibrahim my beloved father, both of you is important to me*
*My siblings that always love me*

*My respective lecturers and tutors those always guide and supporting me*
*My course mates those are always challenging and helping me*

## ACKNOWLEDGEMENTS

Bismillahirrahmanirrahim. Alhamdulillah, thankful to Allah S.W.T for giving me enough time, good health, spirit, and patient in order to complete this project research. With the mercifulness from Allah, therefore I can produce a lot of useful idea as well as learning many skills and gaining new information throughout this project.

I would like to express my thankfulness to my beloved family, especially to my mother, Madam Rahana binti Mahmud and my father, Mister Husin bin Ibrahim for their prayers, support, patient, and motivation in my study.

I would like thank my Supervisor, Mister Rozaimi bin Abu Samah, for his advices, useful guidance, inspiration, patience and support in effort to complete this project. I am indebted to Universiti Malaysia Pahang (UMP) for their facilities, opportunity and financial aid provided in pursuing my project research. I am also grateful to my panels, Miss Asmida binti Ideris and Miss Nasratun binti Masngut for their brilliant idea, advice and guidance to me.

I also would like to dedicate my appreciation to all the lecturers and associates of Faculty of Chemical and Natural Resources Engineering (UMP) that involve in this project for their invaluable time, guidance and advice especially Madam Chua @ Yeo Gek Kee and also post graduate students, Hidawati, Aini and Azrina. Without their cooperation and sacrifices, this research will not be able to complete and published. Last but not least, I would also like to thank to my lab partner, Azimah, Kamal and Sa'adah who have accompanied me throughout this project. Thank you very much.

# ABSTRACT

The crude culture that contains cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) from *Bacillus* sp. TS1-1 has been partially purified by centrifugation and cross-flow filtration. Initial 242.74 U/ml of CGTase was detected in the culture after 24 hours of incubation. The crude supernatant obtained after centrifugation for 5000rpm, 5 minutes and 4$^{o}$C was subsequently filtered at 15$^{o}$C through cross-flow filtration using Kvick Lab cross-flow system. Two cassettes were used with molecular weight cut off of 50K and 10K. The retentate from 50K cassette was further filtered through 10K cassette. Each permeate and retentate from each cassette were tested for CGTase activity. Using cross-flow filtration, the crude enzyme was purified 2.33 fold. The crude and partially purified enzyme was then subjected to gel electrophoresis (SDS-PAGE) to determine the enzyme molecular weight. The partial purified enzyme especially at retentate 50K suggested an initial size of CGTase between 46 to 88 kDa. The crude and partial purified CGTase was then assayed using phenolpthalein method with slight modification for the determination of CGTase optimum pH, pH stability, optimum temperature and thermal stability. Based on this work, the optimum temperature for activity was at 40$^{o}$C and 80$^{o}$C, the thermal stability was from 40-70$^{o}$C and from 60-90$^{o}$C; the optimum pH for activity was at pH6 and pH10; the pH stability was from pH4 to 6.

# ABSTRAK

Kultur mentah yang mengandungi cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) daripada *Bacillus* sp. TS1-1 telah ditulenkan secara separa menggunakan kaedah pengemparan dan penyaringan aliran melintang. Sebanyak 242.74 U/ml CGTase telah dikesan dalam kultur selepas 24 jam pengeraman. Supernatan mentah yang diperolehi selepas pengemparan pada 5000rpm, 5 minit dan 4$^{o}$C kemudiannya disaring melalui penyaringan aliran melintang pada 15$^{o}$C menggunakan sistem penyaring Kvick Lab. Dua blok penyaring bersaiz rongga 50K dan 10K bagi tujuan penyaringan tersebut. Baki kultur daripada blok penyaring 50K seterusnya disaring menggunakan blok penyaring 10K. Setiap hasil dan baki daripada setiap blok penyaring kemudian diuji untuk menentukan aktiviti CGTase. Menggunakan kaedah penyaringan aliran melintang ini, kultur mentah telah ditulenkan sebanayak 2.33 kali ganda. Enzim mentah dan enzim separa tulen kemudiannya dianalisis menggunakan elektroforesis gel (SDS-PAGE) untuk menentukan jisim molar enzim. Enzim separa tulen terutama daripada baki penyaring blok 50K menunjukkan saiz awal CGTase adalah antara 46 hingga 88 kDa. CGTase mentah dan separa tulen seterusnya dicerakinkan menggunakan kaedah Fenoftalein dengan sedikit pengubahsuaian untuk menentukan pH optima, kestabilan pH, suhu optima dan kestabilan suhu bagi CGTase. Berdasarkan kajian ini, suhu optima adalah pada 40$^{o}$C dan 80$^{o}$C dan kestabilan suhu adalah pada 40-70$^{o}$C dan dari 60-90$^{o}$C; pH optima bagi CGTase adalah pada pH6 dan pH10; kestabilan pH adalah pada pH4 – 6.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF SYMBOLS

| | | |
|---|---|---|
| α | - | alpha |
| β | - | beta |
| γ | - | gamma |
| CD | - | cyclodextrin |
| CGTase | - | cyclodextrin glucanotransferase |
| h | - | hour |
| K | - | kilo Dalton (molecular weight cut off) |
| kDa | - | kilo Dalton |
| mg | - | miligram |
| mM | - | miliMolar |
| kg | - | kilogram |
| mol wt | - | molecular weight |
| µl | - | microliter |
| µmol | - | micromole |
| ng | - | nanogram |
| nm | - | nanometer |
| v/v | - | volume per volume |
| v/w | - | volume per weight |
| OD | - | optical density |
| pI | - | isoelectric point |
| rpm | - | revolution per minute |
| SDS-PAGE | - | sodium dodecycl sulphate polyacrylamide gel |
| U | - | unit (enzyme activity) |
| UV | - | ultraviolet |
| V | - | volt |

# LIST OF APPENDICES

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

Cyclodextrin glucanotransferase (EC 2.4.1.19) (CGTase), is an extracellular enzyme. It degrades starch to form cyclodextrins (CDs). The important sources of CGTases are bacteria. The first reported source of CGTases is *Bacillus macerans*. A variety of bacteria that have been determined as CGTase producers are aerobic mesophilic bacteria, aerobic thermophilic, anaerobic thermophilic and aerobic alkalophilic bacteria (Tonkova, 1998).

Major producers of CGTases are *Bacillus* sp. especially aerobic alkalophilic types. Other psychrophilics, mesophilic and thermophilic microorganims that have been identified able to produce CGTase enzymes are *Bacillus stearothermophilus*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* 19-1, *Brevibacterium* sp. and hyperthermophilic archaea-bacteria (Mahat *et al.,* 2004).

## 1.2 Problem Statement

The isolation of pure enzymes also allows active site studies to be carried out on the homogeneous protein, the characteristics of the enzyme such as kinetic

parameters, optimum temperature and pH stability, the effects of ions and crystallization of the enzyme for X-ray crystallographic analysis.

## 1.3 Objective

The objective of this research is to characterize partially purify cyclodextrin glucanotransferase (CGTase) from *Bacillus sp*. TS1-1.

## 1.4 Scope of Study

The scopes of this study are as follows

i. To produce crude CGTase.
ii. To purify CGTase by centrifugation and cross-flow filtration.
iii. To identify pH and temperature effects on stability and activity of the enzyme.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Cyclodextrin Glucanotranferase

Cyclodextrin glucanotransferase (EC 2.4.1.19) (CGTase) degrades starch to form cyclodextrins (CDs) through an intramolecular reaction (cyclization). In this specific reaction, closed circular structures are form when the starch is cleaved and the ends are joined together. In addition, in order to catalyzing the reaction on starch and CDs, CGTase is also involved in intermolecular transglycosylation that involves coupling and disproportionation reactions as well as the hydrolytic action.

There are three different types of CDs which mainly exist: α-CD, β-CD and γ-CD, according to the major CD produced (Rahman *et al.,* 2006). The torus-shaped cyclodextrins have hydrophobic CH groups on the inside and hydrophilic hydroxyl groups on the outside of the ring. Del-Rio *et al.* (1997) has proposed that the CGTase play a biological role where it works in concert with a-amylases for the efficient saccharification of starch.

The Cyclodextrins are natural cyclic oligosaccharides with doughnut-shaped structure possessing hydrophilic surface and hydrophobic central cavity (Fig. 2.0). Due to this unordinary structure CDs are able to form inclusion complexes with

different guest organic and inorganic molecules and also can change physical and chemical properties of the encapsulated guest compound (Szejtli, 2004).

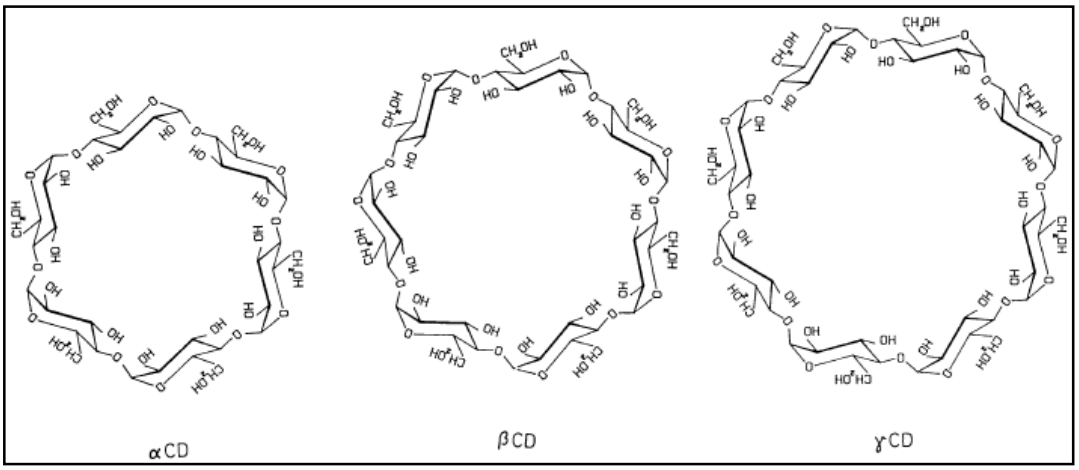


**Figure 2.1**: Molecular models of α-, β- and γ-CD (Szejtli, 2004).

The origin of the CGTase and the reaction conditions are the two factors that influenced the different of the yields and ratios of α-, β- and γ-CD produced from starch. Commonly, different starches is converted into a mixture of α-, β- and γ-CD by various CGTase but the ratios of α-, β- and γ-CD produced are different. CGTases that can synthesize predominantly one type of CD has great commercial importance. This is different from a separation of one type of CDs from the products mixture, where it is time-consuming, costly and tedious (Sian *et al*., 2005).

Production of CGTase can be optimized by manipulating physio-environmental factors such as the nutrient concentrations and compositions of the production media. Media optimization using statistic experimental design has been cited by many researchers in optimizing either biomass growth, enzymes, certain extracellular proteins and bioactive metabolites (Mahat *et al*., 2004).

Typical modes of CGTase production were conducted by Mahat *et al*. (2004) initially by using shake flash culture and submerge fermentation utilizing selective.

Various types of medium composition mainly carbon and nitrogen source concentration, inoculums size, pH and temperature for fermentation production of CGTase had been studied by Gawande *et al*. (1998), Gawande *et al*. (1999) and Stefanova *et al*. (1999).

Applications of CDs are popular and extensively used in industries such as pharmaceutical, toiletries, agricultural, cosmetic, chemical and food. CDs have several behaviors such as increasing the solubility and stability, reducing volatility, controlling release of drugs and masking odors and tastes (Sian *et al*., 2005).

## 2.2 Purification of enzyme

Enzyme purification is an extraction of a single enzyme or protein from examples like cells, tissues which may contain more than 1000 different proteins and lots of other biomolecules. There are several objectives of the purification of enzymes. Firstly is to study reactions, kinetics, regulation and others. Secondly is to understand the deviations in normal metabolism or regulation processes, due to abnormal enzymes. Thirdly is to make a rational design of drugs possible, based on the 3D-structure of a protein. Also through enzymes purification, we can identify which enzymes have themselves an added value, as biocatalysts (proteases, lipases, glucose isomerase), therapeutics (insulin, interleukins) and others.

There were various types of enzymes had been purified as reported by previous researchers. The extracellular endoinulinase from *Xanthomonas oryzae* No. 5 which converts inulin into inulooligosaccharides was purified by Cho *et al.* (2002), from the culture broth by ammonium sulphate precipitation, followed by column chromatography on Phenyl-Sepharose and DEAE-Sephacel. The enzyme was purified 29-fold with a yield of 5.5% from the starting culture broth. The purified enzyme gave a single band on polyacrylamide gel electrophoresis, and its molecular

weight was estimated to be 139 kDa. The specific activity of the purified enzyme was 1372 U/mg.

Li and Peeples (2004) was investigated a purification of a recombinant, thermostable α-amylase (MJA1) from the hyperthermophile, *Methanococcus jannaschii*, in the ethylene oxide–propylene oxide random copolymer (PEO–PPO-2500)/$(NH_4)_2SO_4$, and poly(ethylene glycol) (PEG)/$(NH_4)_2SO_4$ aqueous two-phase systems. In the purification, MJA1 partitioned in the top polymer-rich phase, while the remainder of proteins partitioned in the bottom salt-rich phase. It was found that enzyme recovery of up to 90% with a purification factor of 3.31 was achieved using a single aqueous two-phase extraction step.

Tannase (tannin acyl hydrolase EC 3.1.1.20) produced by *Aspergillus awamori nakazawa* was purified and characterized by Mahapatra *et al.* (2005). The acetone-precipitated fraction was further purified using HPLC (GF-250 column with 4.5 mm x 250 mm, 4 mm pore size) at fixed flow rate; 1 ml/min. The solvent system used to elute the protein based on molecular size was 0.2 M acetate buffer (pH 5). Further purification of the partially purified (acetone precipitated fraction) was achieved by using GFC (using G-100 Sephadex) column. HPLC of the partially purified (acetone precipitated) tannase from the new isolate showed a single major peak and the elution time was 6.8 min.

Shibusawa *et al.* (2007) has conducted a study where a histone deacetylase from *Escherichia coli* cell-lysate was purified by counter-current chromatography (CCC) using aqueous two-phase system. Aqueous–aqueous two-phase (AATP) systems composed of polyethylene glycol (PEG) (molecular mass, *M*r:1000–8000) and dextran (*M*r:40,000) were evaluated for purification of maltose binding protein tagged-histone deacetylase (MBP-HDAC). CCC purification of an MBP-HDAC was demonstrated with a 7.0% PEG 3350–10% dextran T40 system containing 10mM potassium phosphate buffer at pH 9.0. The collected fractions containing target protein were analyzed by an HPLC-based *in vitro* assay and then by sodium dodecyl

sulfate polyacrylamide gel electrophoresis. MBP tag was digested from fusion HDAC during the CCC separation and native HDAC was purified by one-step operation with well preserved deacetyl enzyme activity.

The effect of several metal ions and calcium on purified paraoxonases (PON1 and PON3) from rat liver was studied by Pla *et al.* (2007). In this study, PON1 and PON3 were purified by hydroxyapatite adsorption, chromatography on DEAE-Sepharose CL-6B and non-specific affinity chromatography on Cibacron Blue 3GA. Chromatography on Cibacron Blue rendered two separated peaks: M1 containing PON3 and M2 that contained PON1. PON1 was then further purified by anion exchange on Mono Q HR 5/5. SDS-PAGE of the final preparation indicated a single protein-staining band at 45 kDa. This enzyme was purified 415-fold to apparent homogeneity with a final specific activity of 1370 μmol/(min mg) and an overall yield of 6%. The pooled fractions from Cibacron Blue containing PON3 were chromatographed twice on DEAE-cellulose and a final affinity chromatography step was applied on Concanavalin A-Sepharose. The purity checked by SDS-PAGE showed a single band at about 43 kDa. The overall purification factor was about 177 with a final specific activity of 461μmol/(min mg) and a yield of 0.4%.

Chen *et al.* (2009) had purified and characterized exo- and endo-inulinase from *Aspergillus ficuum* JNSP5-06. Three exoinulinases (Exo-I, Exo-II, and Exo-III) and two endoinulinases (Endo-I and Endo-II) were purified from culture broth of *A. ficuum* JNSP5-06. The purification methods involved were ammonium sulphate precipitation, DEAE-cellulose column chromatography, and Sepharose CL-6B column chromatography. The molecular weights of Exo-I, Exo-II, Exo-III, Endo-I and Endo-II were determined to be 70 kDa, 40 kDa, 46 kDa, 34 kDa, and 31 kDa, respectively. The results from thin-layer chromatography analysis of the hydrolysis products of inulin by four active fractions (A,B,C,D) indicated that the fraction of A, B, and C were exo-type inulinases, whereas D was an endo-type inulinase. It was also found that the I/S ratio of D toward inulin and sucrosewas higher than those of A, B, and C.

Wang *et al.* (2009) successively purified a halostable cellulase from *Salinivibrio* sp. strain NTU-05 using ion exchange chromatography and gel filtration chromatography using the Fast Protein Liquid Chromatography (FPLC) system (Pharmacia Biotech). The crude enzyme obtained as a cell-free supernatant was precipitated using ammonium sulfate to 80% saturation. The protein was purified by ion exchange resources Q chromatography and Sephadex G-200 gel filtration chromatography. The purified enzyme cellulase exhibited 32.4 U/mg specific activity. Overall levels of recoveries and purification of cellulase was observed to be 18.9% with 29.5-fold. The purified enzyme showed a single protein band on SDS-PAGE with an estimated molecular mass of 29 kDa. The zymogram of the cellulase exhibited a significant activity band that corresponded to 29 kDa.

## 2.3 Purification of Cyclodextrin Glucanotransferase

There were several studies that had been reported on the purification of CGTase. Kim *et al.* (1998) had purified and characterized a CGTase from *Paenibacillus* sp. F8. The molecular weight was estimated to be 72 kDa by SDS-PAGE. The initial production ratio of α-CD, β-CD, γ-CD and δ-CD from soluble starch was 0.09:1:0.25:0.14. Prolonged incubation times resulted in a decreased ratio of δ-CD and, to lesser extent, of γ-CD and an increased ratio of α-CD and β-CD compared to the other CD. Coupling experiments showed that δ-CD was more easily degraded by from *Paenibacillus* sp. F8. CGTase compared to α-, β- and γ-CD.

Sian *et al.* (2005) successively purified a CGTase from Bacillus sp. G1 by ammonium sulphate precipitation, and affinity chromatography on α-CD (epoxy)-Sepharose 6B column. SDS-PAGE showed that the purified CGTase was homogeneous and the molecular weight of the purified CGTase was about 75kDa. The molecular weight of the enzyme that was estimated by gel filtration under native condition was 79kDa. In cyclodextrin production, tapioca starch was found to be the

best substrate used to produce CDs. The enzyme produced α- and β-CD in the ratio of 0.11:0.89 after 24 h incubation at 60$^{o}$C, without the presence of any selective agents.

A CGTase from alkalophilic *Bacillu*s sp. 7-12 was purified by Cao *et. al* (2005) by ammonium sulphate precipitation, DEAE-cellulose column chromatography and Sepharose CL-6B column chromatography. The enzyme thus obtained consisted of a single band that did not dissociate into subunits by SDS-PAGE. For cyclodextrin production, up to 34% conversion to cyclodextrins was obtained from 10% starch. The enzyme produced α-, β- and γ-CD in the ratio of 0.26:1:0.86.